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Imagestream detection and characterisation of circulating tumour cells - a liquid biopsy for Hepatocellular Carcinoma?

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Hepatocellular cancer (HCC), Chronic liver disease (CLD), portal vein thrombosis (PVT), extra-hepatic disease (EHD), Eastern Cooperative Oncology Group (ECOG) Performance status (PST), circulating tumour cells (CTCs), red blood cells (RBCs), white blood cells (WBCs), epithelial cell adhesion molecule (EpCAM), cytokeratin (CK), alphafetoprotein (AFP), glypican-3 (GPC-3), DNA-dependent kinase (DNA-PK), Asiaglycoprotein receptor (ASGPR), Isolation by size of epithelial cell (ISET), Hazard ratio (HR), body mass index (BMI), red blood cells (RBCs), white blood cells (WBCs), Type 2 diabetes mellitus (T2DM),

Barcelona Clinic Liver Cancer (BCLC), time to progression (TTP), alcohol related liver disease (ARLD), non-alcoholic fatty liver disease (NAFLD), primary biliary cirrhosis (PBC), autoimmune hepatitis (AIH), drug eluting bead-transarterial chemoembolization (DEB-TACE), selective internal radiation therapy (SIRT), European Association for the Study of the Liver (EASL), European Organisation for Research and Treatment of Cancer (EORTC)

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Author contributions

RP, AVB, NJC, DJ and HLR conceived the study. LO, CEW and CH carried out experimental work and LO, CEW, CH, DJ and HR contributed to experimental design. LO, JGO, SM and

HR recruited patients and generated clinical data. The paper was written by LO, DJ and HR. All authors have read and edited the manuscript.

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Abstract

Background: The lack of progress in developing and delivering new therapies for hepatocellular carcinoma (HCC) is in part attributed to the risk related avoidance of tumour biopsy at diagnosis. Circulating tumour cells (CTCs) are a potential source of tumour tissue that could aid biological or biomarker research, treatment stratification and monitoring.

Methods: An imaging flow cytometry method, using immunofluorescence of cytokeratin, EpCAM, AFP, glypican-3 and DNA-PK together with analysis of size, morphology and DNA content, for detection of HCC CTCs was developed and applied to 69 patient and 31 control samples. The presence of CTCs as a prognostic indicator was assessed in multivariate analyses encompassing recognised prognostic parameters.

Results: Between 1 and 1642 CTCs were detected in blood samples from 45/69 HCC patients compared to 0/31 controls. CTCs positive for the epithelial markers cytokeratin and EpCAM were detected in 29% and 18% of patients respectively, while an additional 28% of patients had CTCs negative for all markers other than size and evidence of hyperploidy. CTC number correlated significantly with tumour size and portal vein thrombosis (PVT). The median survival of patients with >1 CTC was 7.5 months versus >34 months for patients with <1 CTC ($p < 0.001$, Log rank), with significance retained in a multivariate analysis (HR 2.34, 95% CI 1.005-5.425, $p=0.049$) including tumour size and PVT.

Conclusions: The use of multiple parameters enhanced HCC CTC detection sensitivity, revealing biological associations and predictive biomarker potential that may be able to guide stratified medicine decisions and future research. (Word count, 247)

Lay summary

Characteristics of tumour tissues can be used to predict outcomes for individual patients with cancer, as well as help to choose their best treatment. Biopsy of liver cancers carries risks, however, and is usually avoided. Some cancer cells enter the blood, and although they are very rare, we have developed a method of finding and characterising them in patients with liver cancer, which we hope will provide a low risk means of guiding treatment.

Introduction

Hepatocellular Carcinoma (HCC) complicates chronic liver diseases (CLD) and, owing to a combination of incidence, late stage presentation and lack of therapeutic options, is the second commonest cause of cancer related death globally [1]. The predominant causes of CLD include hepatitis B and hepatitis C, although the prevalence of obesity and alcohol excess are having a major impact, with mortality continuing to rise despite advances in antiviral therapy [2]. The presence of associated CLD severely limits the role of traditional anti-cancer cytotoxic agents in patients with HCC. While 'targeted' medical therapies have emerged for other cancer types in recent years, advances for patients with HCC have been hampered by both a failure to identify or successfully 'drug' the key drivers of hepatocarcinogenesis, as well as an inherent lack of biological markers to support stratification of both traditional and emerging therapies. This handicap is attributed to the lack of tissue biopsy as standard diagnostic practice for patients with HCC. In the majority the diagnosis can be confidently made using imaging criteria, avoiding biopsy and the associated risks of haemorrhage and tumour seeding [3]. There is a need, therefore, for HCC biomarkers other than those acquired by standard staging or tissue biopsy, that can be used to assist treatment stratification and to determine prognosis. The development of a 'liquid biopsy' enabling evaluation at multiple time points would be a major advance.

First described by Thomas Ashworth in 1869, circulating tumour cells (CTCs) are those cells that have detached from a primary or secondary tumour and can be detected in the peripheral circulation [4]. Although the potential significance of these cells has long been realised, clinical implementation has been slow owing to a lack of cancer-specific biomarkers and difficulties detecting these rare cells (estimated frequency ≤1 CTC/ml of blood) against a high background of haematopoietic cells and blood components. Recent technological advances have started to have an impact in metastatic breast, prostate and colorectal cancers, where

CTCs positive for epithelial biomarkers such as cytokeratin (CK) and epithelial cell adhesion molecule (EpCAM) have been detected using immunogenic capture with the CellSearch® system (Janssen Diagnostics) and enumeration of CTCs shown to be a useful predictor of prognosis in terms of median overall survival and time to progression [5-7].

Currently, there are limited studies detecting and characterising CTCs in HCC, with no standardisation of methods and techniques. CTC detection of ≥1 or 2 CTC using the CellSearch system is reportedly in the region of 30-35%, although positive cells have occasionally been detected in controls [8-10]. An alternative size based filtration technique (Isolation by Size of Tumour cells or ISET®) identified cells presumed to be CTCs during tumour resection in a study published in 2000, but with a sensitivity that could only detect these cells as 'microemboli' prior to surgery (3 of 7 patients), rather than as single cells [11]. More recently, Morris and colleagues evaluated the ISET and CellSearch methods in patients with HCC, confirming a CellSearch sensitivity similar to that previously reported (15/50; 28%) compared to 100% (19 of 19 patients) for ISET [9]. While ISET sensitivity was promising, specificity was dependent on size only, with no associations in the small number of cases studied with tumour stage or outcome [9]. Other researchers have explored the use of positive immunomagnetic selection with mesenchymal markers in combination with alternative liver specific markers such as asiaglycoprotein receptors (ASPGR), or in-situ hybridisation to detect molecular aberrations (TP53 deletion, HER-2 amplification) [12, 13]. As with the other methods, the clinical relevance of detection of these populations of cells in patients with HCC remains to be determined.

We have previously outlined a method for the detection and characterisation of CTCs using the Imagestream (Amnis®, EMD Millipore) imaging flow cytometer [14]. Here we report our subsequent study in patients with HCC, using a combination of image, size and fluorescently detected biomarkers. These included the epithelial biomarkers EpCAM and cytokeratin, HCC specific biomarkers AFP and glypican-3 (GPC3), as well as DNA-PK – a candidate biomarker

for treatment stratification in HCC [15]. We have detected at least 1 CTC in 65% (45/69) of patients, showing significant correlations between CTC number and adverse tumour characteristics. Furthermore, the presence of CTCs was an independent predictor of poorer survival.

Patients and Methods

Cell culture

A panel of 6 authenticated HCC cell lines (LGC standards) were assessed for cell area. HepG2, Hep3B, Huh-7 and PLC/PRF/5 were cultured in Dulbecco's modified eagle's medium (DMEM) with 15mM HEPEs, pyridoxine and $NaHCO_3$ (Sigma-Alrich, UK) supplemented with 10% foetal bovine serum (FBS), (Gibco, UK) and 2.5% 200mM L-Glutamine solution (Sigma-Alrich, UK). SNU182 and SNU475 cells were maintained in RPMI 1640 medium (Sigma-Aldrich, UK) supplemented with 10% FBS. Cells were maintained in the growth phase and passaged when ~70% confluent. Regular mycoplasma testing was performed using the MycoAlert mycoplasma testing kit, Lonza, USA.

Assessment of biomarker expression in HCC cell lines

HCC cell lines were assessed for the expression of biomarkers using the Imagestream. The SJSA1 human osteosarcoma bone fibroblast cell line was used as a negative control when assessing the expression of epithelial biomarkers. A population of round single cells was gated by using a scatterplot of brightfield aspect ratio against area. From this population in focus cells were selected by creating a histogram of the root mean square feature which detects differences in intensity between adjacent pixels. A gate of in focus cells was created and assessed for the mean pixel intensity of biomarkers of interest. Numerical data on different cell populations was exported to GraphPad Prism Software, version 6 (GraphPad Prism Software Inc, California, USA).

Patient blood samples

As part of a pilot project exploring the utility of the Imagestream in patients with HCC, blood samples were collected from patients attending the Newcastle upon Tyne Hospitals NHS Foundation Trust between November 2012 and January 2015. With the initial aims of optimising enrichment and analysis methodologies, as well as assessing a panel of CTC

biomarkers, there was no patient selection criteria other than an established diagnosis of HCC, based on EASL-EORTC guidelines [3], and patient consent, aiming to collect 1 sample per week. Patients with an active malignancy at another site were excluded. Control samples were from 15 healthy volunteers and 16 patients with cirrhosis without cancer. Ethical approval was obtained through the Newcastle Hepatopancreatobiliary and Gastroenterology Research Tissue Biobank. The patient dataset included age, sex, body mass index (BMI),blood count and biochemistry, as well as tumour staging on cross-sectional imaging - tumour size, portal vein thrombosis (PVT), presence or absence of extra-hepatic disease (EHD). ECOG Performance Status score, Child-Pugh score and Barcelona Clinic for Liver Cancer (BCLC) stages were recorded, as were treatment regimens. Follow-up was until 15th September 2015, recording whenever possible the time to clinical progression and radiological progression using mRECIST (modified Response Evaluation Criteria in Solid Tumors) criteria [3], as well as date of death, from the date the blood sample for CTC analysis was taken.

Patient sample processing

Patient blood samples were collected in either EDTA (BD Vacutainer) or CellSave preservative tubes (Janssen Diagnostics). EDTA tubes were transported on ice to the laboratory where they were processed immediately. CellSave tubes were transported at ambient temperature and cells were left to fix for at least 1 hour. A total of 4 ml blood was processed from EDTA samples and 8 ml from CellSave tubes and cell number for each sample expressed as number of cells per 4 ml whole blood. Patient samples were processed according to a previously developed method which reported recovery of 57.3, 49.2 and 59.0 % of cells spiked into whole blood at densities of 500, 50 and 5 per ml respectively [14]. Unfortunately it was not possible to assess in-study sample reproducibility. Briefly, samples were blocked using BSA solution and FcR block (MACS®, Miltenyi) to prevent unspecific antigen reactions. The samples were depleted of red blood cells (RBCs) by incubation with BD Phosflow™ lyse/fix red cell lysis buffer (BD Biosciences). The sample was then immunomagnetically depleted of white blood

cells (WBCs) using a CD45 depletion kit and BigEasy magnet (StemCell Technologies). The remaining cell suspension was then stained with a series of immunofluorescent antibodies including: pan-CK (CK -4, -5, -6, -8, -10, -13 and -18), EpCAM, AFP, GPC3, DNA-PK and CD45 (**Supplementary Table S1**). Nuclei were stained with either DRAQ5 or DAPI. Following staining, cells were washed and re-suspended in PBS prior to processing through the Imagestream. Analysis was performed using the IDEAS software (Amnis, Seattle) and CTCs were identified on the basis of brightfield morphology, size, antigen expression, nuclear signal and the absence of CD45 expression. Objects that did not meet these criteria but had a consistent cell morphology and lack of CD45 expression were also identified. Cell area was calculated by creating a custom brightfield mask based on the standard brightfield mask eroded by 3 pixels to allow closer fit to the brightfield image. A new area feature of this mask was created and area values calculated in μm^2.

Statistical Methods

Statistical analyses were carried out using SPSS, version 21 (SPSS Inc. Chicago, USA) licensed to Newcastle University and GraphPad Prism Software, version 6 (GraphPad Prism Software Inc, California, USA). Bivariate associations were Pearson or Spearman's Rho correlations for parametric or non-parametric data respectively. Differences between groups of continuous variables were assessed by t-test (parametric data) or Mann-Whitney (non-parametric data) tests. Differences between categorical variables were assessed by Pearson Chi square, or Fisher's exact tests approximated using a Monte Carlo approach where cells within a contingency table of greater than 2 x 2 contained low numbers (<5). A p value of <0.05 was considered significant. Survival was recorded as months from blood sampling for CTC detection to 15/09/2015. Differences in cumulative survival were determined using the Kaplan-Meier method and a Log-Rank test. The Cox proportional hazards-regression model was used to identify parameters associated with survival. Factors initially considered by univariate

analysis included age, body mass index (BMI), T2DM, number of tumours, size of largest tumour, presence of PVT or EHD, ascites or encephalopathy, serum alpha-fetoprotein (AFP), serum albumin, serum bilirubin, prothrombin time, performance status and treatment. A cutoff of $p < 0.05$ was used to select variables entered into the multivariate model.

Results

Biomarker heterogeneity between and within HCC cells

We initially characterised biomarker staining profiles of HCC cells lines with antibodies to EpCAM, CK, AFP and GPC3. Suspended cells trypsinised, suspended and stained prior to analysis by the Imagestream. Round single cells were identified by creating a scatterplot of brightfield aspect ratio against area as described in the methods. After additional gating to exclude double cells or debris, the in focus single cell population was assessed for the mean pixel intensity of the biomarker of interest, as an estimate of biomarker expression. Heterogeneity was observed between the HCC cell lines studied, as shown in **Figure 1A-D**. Hep3B cells for example had the highest expression of EpCAM, AFP and GPC3, but the lowest expression of cytokeratin. SNU182 cells had high levels of cytokeratin and EpCAM, with relatively low levels of GPC3. PLC/PRF/5 and SNU475 had particularly low levels of GPC3. More striking than the differences between the cell lines, was the differences within the HCC cell lines. The range of pixel intensity included negative cells in all cases, including an EpCAM negative subpopulation in Hep3B cells. While these data highlight the importance of using more than one epitope for CTC detection, they also highlight the variability of expression even within cultured cell populations arising from the same tumour. Ideally, therefore, CTC detection should be based on additional characteristics less subject to change.

Imagestream discrimination of CTC from white blood cells on the basis of size

During analysis of both HCC cell lines and patient samples following depletion, data analysis files produced following compensation frequently contained biomarker negative objects >50 μm in diameter. Some of these were clusters of CD45 +ve WBCs escaping depletion. Other larger nucleated cells were CD45 –ve and suspected to be biomarker negative CTCs. There

has been debate as to whether or not CTCs can be identified simply on the basis of size. The ISET filtration enrichment method relies on CTC size, but potentially lacks specificity without additional characterisation of candidate cells. CTCs may also escape retention by this method consequent to their deformability [16]. The Imagestream enables objective characterisation of the size of the high-resolution brightfield images. Thus we assessed the surface area of the brightfield images of the circulating cells, as shown in **Figure 2A**. The surface area of CD45+ve WBCs of our volunteers and patient cohort (mean 88.3 ± 0.20 μm^2) was compared that to the surface area of HCC cell lines and CTCs (**Figure 2B**). The HCC cells were distinguishably larger than WBCs, ranging from HepG2 (219.5 ± 54.1 μm^2) to SNU475 (402.7 ± 2.9 μm^2). The area of single cell images in distinct biomarker negative and positive populations of CTCs was analysed (n=11 and n=7 cases respectively). The average area of biomarker positive CTCs (mean area of 362.2 ± 55.5 μm^2) was comparable to that of HCC cell line cells, with no overlap with the WBC population, and was similar to the area of biomarker negative CTCs (345.2 ± 38.4 μm^2, Figure 2B). Interpatient heterogeneity in area of CTC images, between biomarker positive and negative cells, was also similar in the two patients with CTC counts greater than 100 (Figure S1). Therefore, after gating to exclude doublet cells and debris, CTCs could be detected by Imagestream on the basis of size, even in the absence of classical biomarker expression (**Figure 2A** and **2B**). The DNA assessment of biomarker negative cells confirmed that they were hyperploid, with more DNA than peripheral WBC, strengthening the case for these being tumour in origin.

Imagestream detection of CTCs in 65% of patients with HCC.

The HCC patient cohort described in **Table 1** included 69 patients. Their median age was 73 years, with 71% (49/69) having underlying cirrhosis. Alcohol related and none alcohol related fatty liver diseases were the commonest etiologies, with 11.6% having no evidence of

underlying liver disease. Although the majority (58/69) had preserved liver function classed as Childs Pugh stage of A, 72% (50/69) were classed as BCLC stage C, often on the basis either advanced tumour stage (PVT, EHD) or ECOG performance status of 1 or greater. Arterial intervention or supportive care were the commonest treatments, although 14 patients (20%) underwent potentially curative therapies (liver transplant, tumour resection or tumour ablation). Arterial treatment was typically with transarterial chemoembolization (TACE) with doxorubicin eluting beads, although one patient received selective internal radiotherapy treatment (SIRT) first line and four patients received second line SIRT, treating tumour progression post TACE. In 40 of the 69 cases, the samples for analysis were taken before any therapeutic intervention, while in the remainder the samples were post treatment (median 85 days). CTCs were detected based on brightfield image characteristics and size in 45/69 (65%) HCC patient blood samples compared to 0/31 control samples. Control samples were from 15 healthy volunteers and 16 cirrhotic patients without cancer. The number of CTCs detected in patients was highly variable, with counts ranging between 1-1642 cells per 4 ml blood samples (**Figure 2C**). Eleven patients had previously received curative therapy for other cancers (including lymphoma, breast, prostate, colorectal, oral, basal cell and superficial bladder cancers) without any sign of recurrence after a minimum of 3 years follow-up.

Biomarker expression in HCC CTCs

Inclusion of classical epithelial markers (EpCAM and CK) in the biomarker panel enabled comparisons with CellSearch data in the HCC literature. In addition, AFP was used as an HCC specific biomarker. The expression of candidate biomarkers (GPC3 and DNA-PK) that could be useful in terms of diagnosis and treatment stratification were also explored. As observed with HCC cell lines, expression of the classical epithelial and exploratory biomarkers was highly heterogeneous between patients and within the same patients, with examples of cases shown in **Figure 3A-E**. CK was the most commonly detected biomarker (29%), followed by

DNA-PK (24%), AFP (20%), EpCAM (18%) and GPC3 (12.5%). In total, 37% of cases had CTCs that had positive expression of one or more of the candidate biomarkers, while an additional 28% had CTCs that were negative for all included tumour biomarkers.

Imagestream CTC number was associated with advanced tumour stage

Samples were collected from HCC patients at various disease stages and undergoing different treatment regimens (**Table 1**). In the whole cohort, weak correlations between CTC number and some adverse prognostic features were observed, including a weak correlation with the size of the largest tumour (0.291, p=0.015, Spearman's Rho **Figure 4**). Consequently, the sensitivity of Imagestream CTC detection was explored in cases with tumours below or equal to 2 cm in widest diameter on imaging compared to those with one or more HCC greater than 2 cm. At least 1 CTC per 4 ml blood sample was detected in 9/15 (60%) cases with an HCC <2 cm versus 36/54 (67%) cases with HCC >2 cm, which was not significantly different (p=0.632; Chi square test). Limiting the comparison to cases where greater than 1 CTC per 4 ml were detected, the comparative figures were 5/15 (33%) and 27/54 (50%) (p=0.252; Chi square test). In the whole cohort, there was also a weak correlation between CTC number and the presence of encephalopathy (0.336, p=0.005; Spearman's Rho test). 41/65 patients without encephalopathy had CTCs detected, with a median CTC number of 1, compared to CTCs detected in 4/4 patients who did have encephalopathy, with a median CTC number of 10.

Associations within and between the different biomarker categories of CTC were also explored. The strongest association was between numbers of cytokeratin and AFP positive CTC (0.786, p<0.0001), with highly significant correlations also between numbers of EpCAM

and cytokeratin positive cells (0.497, p<0.0001) and AFP positive cells (0.463, p<0.0001). GPC3 expression correlated with EpCAM (0.405, p=0.024) and no other epithelial biomarker. Taken individually, the numbers of biomarker positive cases were relatively small, without significant correlations with stage of disease. As a group, numbers of biomarker positive CTCs trended toward an association with tumour size (0.236, p=0.05), with a weak negative correlation with serum albumin (-0.354, p=0.003) and weak positive association with the presence of encephalopathy (0.331, p=0.005). The numbers of biomarker negative cells correlated with none of the other biomarkers, but there were indications of an association with numbers of neutrophils (0.240, p=0.049) in the peripheral blood count taken on the same day. In addition, numbers of biomarker negative CTCs were weakly associated with the presence of PVT (0.348, p=0.004; Spearman's Rho test). 12/14 patients with a PVT had CTC detected and in 10 of these cases, CTCs were epithelial biomarker negative.

The presence CTCs was associated with poorer Survival

Associations with overall survival were explored by univariate cox regression, as shown in **Table 2A**. Significant associations included the number of CTCs per 4 ml blood sample (p=0.043), tumour size (p<0.0001) and the presence of either a PVT (p=0.002) or EHD (p<0.0001). CTCs and presence of PVT or EHD were independent of each other in a multivariate analysis (**Table 2A)**. Notably, while the presence or absence of CTC was not significantly associated (p=0.127), survival comparing those individuals with 0 or 1 CTC per 4 ml blood sample (n=37; 54%) to those with >1 CTC per 4 ml blood (n=32; 46%) was markedly different (threshold of >1 was determined by ROC analysis, optimal cut off >1.250, sensitivity 68.75%, specificity 72.97, likelihood ratio 2.544). The median survival was in excess of 34 months in the former group, compared to 7.5 months in the latter (p<0.0001, Kaplan Meier with Log Rank test, **Figure 5A**). This 'cut off' remained significant in a multivariate analysis

(p=0.049, HR 2.34, 95% CI 1.005 – 5.425) (**Table 2B**). Disease progression was not protocol driven in this case series and time to progression (TTP) was estimated based on imaging performed as part of standard care, or clinical deterioration changing the BCLC stage. Four patients died without documented progression and were excluded from TTP analyses. Similar to the survival data, TTP between those with or without >1 CTC per 4 ml blood was significantly different (p=0.006 Kaplan Meier with Log Rank test, **Figure 5B**).

Analyses pre and post treatment were limited in this small case series, as was the possibility to explore associations within specific biomarker or treatment groups. Considering only the 40 cases where samples were taken at diagnosis and staging, CTC >1 per 4 ml blood, tumour size, PVT and EHD were each associated with poorer survival, although only the presence of PVT was significant in a multivariate analysis (data not shown). In the post treatment group (n=29), TTP and survival were significantly shorter in those with CTC >1 per 4 ml blood as shown in **Figure 5C** and **D**. Both CTC number and tumour size remained independent predictors of poorer survival. Treated as a categorical variable, only CTC >1 per 4 ml blood was independently associated with poorer survival (HR 6.16, CI 1.71-22.33, p=0.006). In the treated group, nineteen of the individuals received arterial therapy and similarly, CTC >1 per 4 ml blood post treatment was the only factor significantly associated with a poorer survival (p=0.022, HR 5.02, 95% CI 1.26-19.93).

Additional observations in blood samples from patients with HCC

There were a range of other objects that were detected by the Imagestream that may have gone undetected using other methods. In some patient samples, CTCs appeared to be travelling in aggregates (**Figure 6A**). This has been previously described by researchers using the ISET method of CTC isolation. The survival outcome of patients with aggregates detected was comparable with that of patients with more than 1 CTC but no aggregates detected (Data not shown). Occasionally, CTCs showed signs of cell division or bi-nucleation

(**Figure 6B**). In some patients, CTCs appeared to have leucocytes attached to them (**Figure 6C**). This is suggestive of a tumour directed immune response and has not been previously described using other methods. The clinical relevance of these observations are as yet unknown. Cells significantly larger than CTC (895.9 ± 41.9 μm^2 versus 255.3 ± 6.9; n=100 paired t-test $p<0.0001$) and with a less circular morphology were detected (**Figure 6D**) in the majority of patients with HCC as well as controls. These were classed as macrophages.

Discussion

Following initial method development, we have previously shown that the Imagestream can be used as a CTC detection platform for the enumeration of CTCs in different cancer types [14]. Here we have taken that work forward in patients with HCC, demonstrating that the Imagestream method detects epithelial biomarker positive CTCs in similar numbers of patients compared to other methods, but that it has several significant advantages. Increasing the number of biomarkers used to characterise cells identified additional CTCs - both in the same patients, but also in additional patients. Furthermore, we have demonstrated that inclusion of a size criterion in combination with absence of CD45 positivity and brightfield image analysis captured all positive biomarker CTCs, but also identified biomarker negative candidate CTCs in an additional 28% of patients. In total, we have identified CTCs in 45/69 (65%) of our patients compared to none of 31 control subjects. Notably, in contrast to other methodologies used to detect CTCs in patients with HCC, Imagestream enumeration demonstrated significant associations with advanced HCC stage as well as independent associations with patient survival.

It has previously been suggested that reliance on epithelial markers for CTC detection may exclude the detection of a proportion of the CTC population that do not express these markers, or have lost expression consequent to the process of epithelial mesenchyme transition (EMT [17-19]. In this study, we have confirmed that not all HCC CTC detected do express the classical epithelial markers EpCAM and CK. Some cells expressed one epithelial marker but not the other, while some expressed AFP and neither cytokeratin nor EpCAM. This could partly explain the low detection rates in studies that have used CellSearch in HCC, as published CellSearch studies have relied on the expression of EpCAM for the immunomagnetic capture of CTCs and CK for their detection [9, 10, 13, 20]. In addition to the

detection of cells that did not express classical epithelial markers however, an additional 28% of patients had objects that were nucleated and cellular in morphology, negative for all biomarkers, but of a size typical of a CTC rather than any other blood cell. While it is difficult to ascertain with absolute certainty that these cells were CTCs, given the complex heterogeneity of HCC and the detection of many biomarker negative cells within HCC cell line populations, identifying additional cells negative for the biomarkers included was not surprising. The weak association of these cells with neutrophils raises the obvious question as to whether these might be white blood cells. This is improbable, given the cells were CD45 negative, dissimilar in both size and morphology from the white cells, with elevated DNA content. Abnormal DNA content has been reported in patients with cirrhotic dysplasia [21] – thus the elevated DNA content does not unequivocally identify cells as malignant. In our series thus far, however, these cells have not been detected in the circulation of any control subject, including 16 cirrhotic patients without cancer. An interesting alternative hypothesis, particularly given the associations between biomarker negative cells, neutrophils and portal vein invasion, is that these biomarker negative objects are CTCs that have undergone EMT. EMT is associated with HCC progression with a suspected role for cancer associated neutrophils driving these processes [22, 23]. The inclusion of an additional biomarker, such as ASPGR - co-expressed in CTC with EMT markers vimentin and twist in association with portal vein invasion [24] - may allay any remaining doubts regarding the origin of these cells in future studies.

In several HCC patients clusters of CTCs were identified as previously reported by researchers using the ISET method [11]. The significance is unknown, but others have suggested that CTCs travelling in clusters may be more likely to evade the immune system and promote metastatic spread by facilitating survival and growth of secondary lesions compared to single cells [25]. Immune interactions between single CTCs and WBCs were observed and these may be CTCs that are actively cleared/deactivated by immune cells and

therefore unable to establish metastases. A counter argument, however, is that CTCs travel surrounded by leucocytes as a form of protection [17] and without larger studies – possibly including further characterisation of the CTC associated white cell population – the relevance of these interactions remains unknown. Imagestream analyses are on fixed cells and future studies will likely benefit from complementary methods, to enable sorting and isolation of living cells, as well as their additional comprehensive characterisation or culture.

In the present study, the principal aim was to develop relevant Imagestream methodologies and explore their utility. We have shown how heterogenous biomarker expression in CTCs in the same patients can be, demonstrating the need for multiple biomarkers and additional criteria such as size and brightfield image to increase the sensitivity and specificity of CTC detection. With this approach we have identified CTCs in 65% of our patients. While numbers of CTCs appeared to increase with tumour size, detection was not limited to large tumours. In this small series we have observed weak associations with features alluding to tumour biology – including peripheral neutrophil count and portal vein invasion – and also shown independent CTC associations with patient survival. Evidently these observations need confirmation in a prospective fashion in predefined patient groups. Furthermore, in larger series of surgically treated patients, it will be important to explore the relationship between CTC biomarker expression profiles with the immunohistochemistry profile of the tumour. However, this paper strongly supports CTC enumeration as a credible liquid biopsy tool. In combination with the development of methods to detect and quantify potential stratification biomarkers, such as c-MET, or pharmacodynamics markers for monitoring treatments, these tools are likely to have a major impact in the future management of our patients with HCC.

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Figure Legends

Figure 1. Heterogenous expression of biomarkers in HCC cell lines

HCC cell lines included HepG2, Hep3B, Huh-7, PLC/PRF/5, SNU182 and SNU475), while SJSA1 osteosarcoma cells were used as a negative control for epithelial biomarkers. Cells were stained with antibodies against A. EpCAM (conjugated to AF488); B. pan-CK (conjugated to PE); C. AFP (conjugated to AF594); D. GPC3 primary antibody and AF488 goat anti-mouse secondary antibody. DAPI was used as a nuclear stain. Cells were imaged using the ImageStream. Single cells were gated based on brightfield area and aspect ratio. In focus cells were selected using the root mean square feature and then assessed for the mean pixel intensity of the respective biomarkers using the IDEAS software. Data analysis was after export to GraphPad Prism. Expression levels of each markers was different between the cell lines (Kruskal Wallis, $p<0.001$ in each case).

Figure 2. CTCs can be distinguished by size and detected in 65% of patients with HCC

Cell area was assessed after creating a brightfield mask, the histogram of brightfield area shown in (**A**)**.** The large peak of smaller area cells is that of single white blood cells. Objects with an increased brigthfield area included doublets of white blood cells, three or more white blood cells and CTCs (**A**). The brightfield area of single in-focus cells, including white blood cells, HCC cell lines (HepG2, Hep3B, Huh-7, PLC/PRF/5, SNU182), biomarker positive CTCs and macropahges is shown in table form (**B**) with values displayed as mean ±SEM. Panel (**C**) shows the number of CTCs per 4 ml of blood after CD45 immunomagnetic depletion in 69 patients with HCC. *number of single cells from representative experiments, **number of patients in which the mean CTC area was assessed, ***number of cells pooled from patient samples.

Figure 3. Heterogenous expression of biomarkers in patients with HCC

CTCs observed in HCC patient samples displayed heterogenous expression of biomarkers, as shown in individual patients. In case (**A**), distinct EpCAM-positive and cytokeratin (CK) - positive CTCs were detected. Cases **B-C** demonstrate CTCs that were positive for different combinations of EpCAM, CK, AFP and DNA-PK, including a CTC doublet positive for EpCAM and DNA-PK in (**C**). A GPC3-positive CTC is shown in (**D**). (**E**) shows biomarker negative CTCs distinguished on cellular morphology, size and DNA content.

Figure 4. CTC number correlates with tumour size

Patients with larger tumours tended to have a greater number of CTC detected (0.291, p=0.015). Black dots represent patients samples taken before any treatment, while white dots are those from patients whose sample was taken post treatment.

Figure 5. Greater than 1 CTC identifies patients with a poorer outcome

In patients with HCC, those with >1 CTC per 4 ml blood had a poorer outcome, with a median survival of 7.5 months compared to >34 months (Kaplan Meier Log-Rank p<0.0001, multivariate analyses in Table 2) (**A**). Time to progression (TTP) first documented, either radiological or clinical, is shown Panel (**B**). Similarly, in the subgroup of patients who had received treatment, the presence of >1 CTC per 4 ml blood was highly significantly associated with poorer survival (**C**) and shorter TTP (**D**).

Figure 6. Additional CTC observations

(**A**) Clusters of EpCAM-positive CTCs. (**B**) EpCAM-positive CTCs that appear to be mitotic. (**C**) CTCs with immune interaction - in this case an EpCAM-positive CTC is surrounded by

CD45-posiitve white blood cells that escaped depletion. (**D**) Cells larger than CTCs, with less circularity, were presumed to be macrophages.

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Author names in bold designate shared co-first authorship.

Table 1. Patient cohort for CTC studies

Table 1			
Cases	All	At presentation	Post-treatment
Number of cases	69	40	29
Age (median)	73	74	69
Liver disease			
None	8	6	2
ALRD	22	13	9
NAFLD	24	12	12
PBC/AIH	9	3	6
Haemochromatosis	4	4	0
Cryptogenic	2	2	0
Cirrhosis (present)	49	25	24
Tumour number	1.9±0.2	1.9±0.2	1.8±03
Tumour size (mm)	58±6	72±9	38±5
PVT (present)	14	9	5
EHD (present)	9	4	5
Encephalopathy (present)	4	2	2
Ascites (present)	6	5	1
Childs Pugh A/B/C	58/8/3	31/6/3	27/2/0
BCLC A/B/C/D	11/5/50/3	4/1/32/3	7/4/18/0
PST 0/1/2/3	21/17/27/4	8/9/19/4	13/8/8/0
AFP (median, kU/L)	14 (≤1-≥50000)	14 (≤1-≥50000)	18.5 (≤1-≥50000)
Albumin (g/l)	38.9±5.4	38.5±5.3	39.5±5.6
Bilirubin (µmol/l)	18.2±15.4	19.0±15.3	17.2±15.8
Prothrombin time (s)	12.6±2.5	12.9±2.7	12.3±2.3
Primary Treatment			
Liver transplant	4	2	2
Resection	3	2	1
Ablation	7	3	4
Arterial treatment	27	7	20
Sorafenib	9	7	2
Supportive care	19	19	0

The cohort of 69 patients studied included 40 recruited during their diagnostic and staging work-up, and 29 patients attending outpatient follow-up post treatment. The majority had either alcohol related liver disease (ARLD) or non-alcoholic fatty liver disease (NAFLD). Other diseases included primary biliary cirrhosis (PBC) or autoimmune hepatitis (AIH). Staging features are shown, including Portal vein thrombosis (PVT), extrahepatic disease (EH), Barcelona Clinic for Liver Cancer (BCLC) stage and ECOG performance status (PST). Ablation was using microwave. Arterial treatment was drug eluting bead transarterial chemoembolization (DEB-TACE) or systemmic internal radiotherapy treatment (SIRT).

Table 2. Predictors of Survival

2A	Univariate Analysis	Multivariate Analysis CTC considered as a continuous variable				2B	Multivariate Analysis CTC considered as a categorical variable		
				95% CI				95% CI	
		p	HR	Upper	Lower	p	HR	Upper	Lower
CTC number 4ml	0.043	0.025	1.002	1.000	1.003	(Not included)			
CTC >1 / 4ml	<0.0001	(Not included)				0.049	2.335	1.005	5.425
Age (median year)	0.217								
BMI	0.474								
T2DM	0.939								
Cirrhosis	0.643								
No of tumours	0.071								
Size of largest (cm)	<0.0001	0.174	1.005	0.998	1.013	0.065	1.007	1.000	1.014
PVT (cat)	0.002	0.005	3.568	1.467	8.679	0.023	2.575	1.140	5.818
EHD (cat)	0.001	0.003	4.163	1.634	10.605	0.122	1.999	0.831	4.807
Ascites	0.061								
Encephalopathy	0.183								
AFP (median)	0.065								
Albumin (g/l)	0.120								
Bilirubin (µmol/l)	0.582								
Prothrombin time	0.566								
PST (cat)	0.127								
Treatment (cat)	0.072								
BCLC stage	0.187								

In the multivariate cox regression analysis, variables with a p value <0.05 on univariate analysis were included. For categorical variables (cat) the comparator was that variable with the best outcome i.e. no portal vein thrombosis (PVT) or extrahepatic disease (EHD. Significance and Hazards Ratio (HR) with upper and lower 95% confidence intervals (CI) are shown.
(A) The presence of EHD, PVT and CTC number were independently associated with survival, although the HR for CTC number was small. (B). When CTCs were considered as a categorical rather than a continuous variable, comparing CTC 0 or 1 to cases with CTC >1, the HR was similar to that for PVT (B).

Figure 1. Heterogenous expression of biomarkers in HCC cell lines

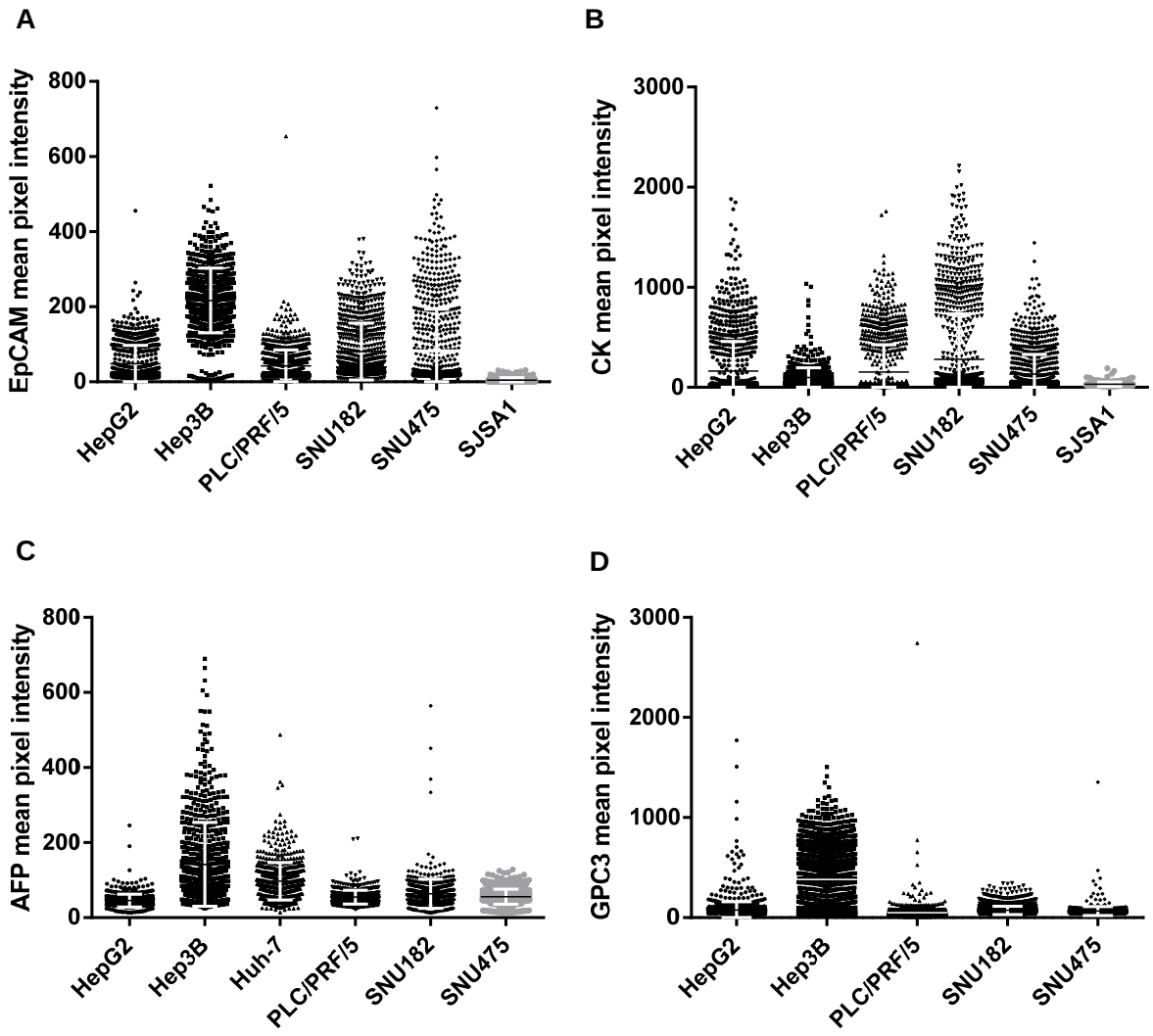
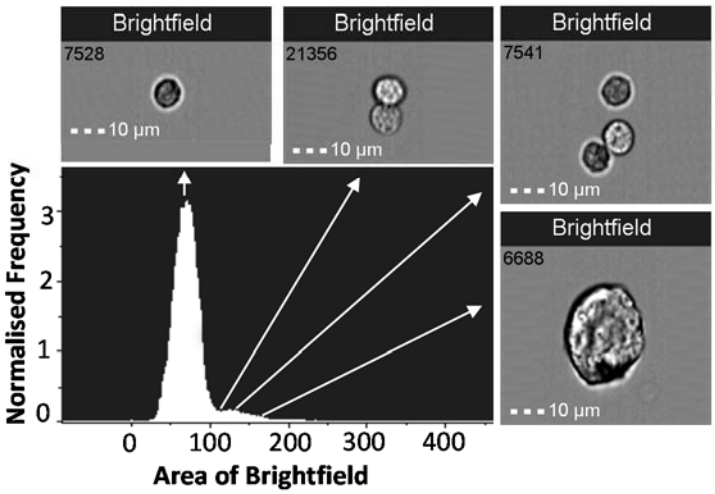


Figure 2. CTC can be distinguished by size and detected in 65% patients with HCC

A



B

Cell type	Surface Area (μ^2)	Number
WBC	88.3 ± 0.2	8000*
HepG2	219.5 ± 2.1	1130*
Hep3B	243.0 ± 1.5	2182*
Huh-7	268.7 ± 2.1	1777*
PLC/PRF/5	244.4 ± 1.3	1678*
SNU182	388.9 ± 1.8	2343*
SNU475	402.7 ±2.9	1850*
CTC		
Marker pos	362.2± 55.5	7**
Marker neg	345.2± 38.4	11**
Macrophages	895.9 ± 41.9	100***

C

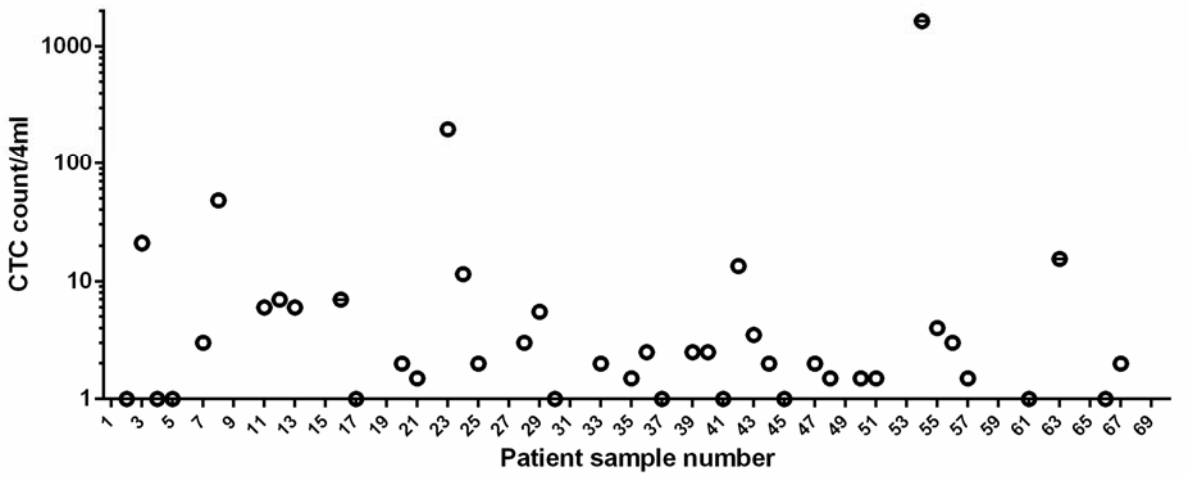


Figure 3. Heterogenous expression of biomarkers in patients with HCC

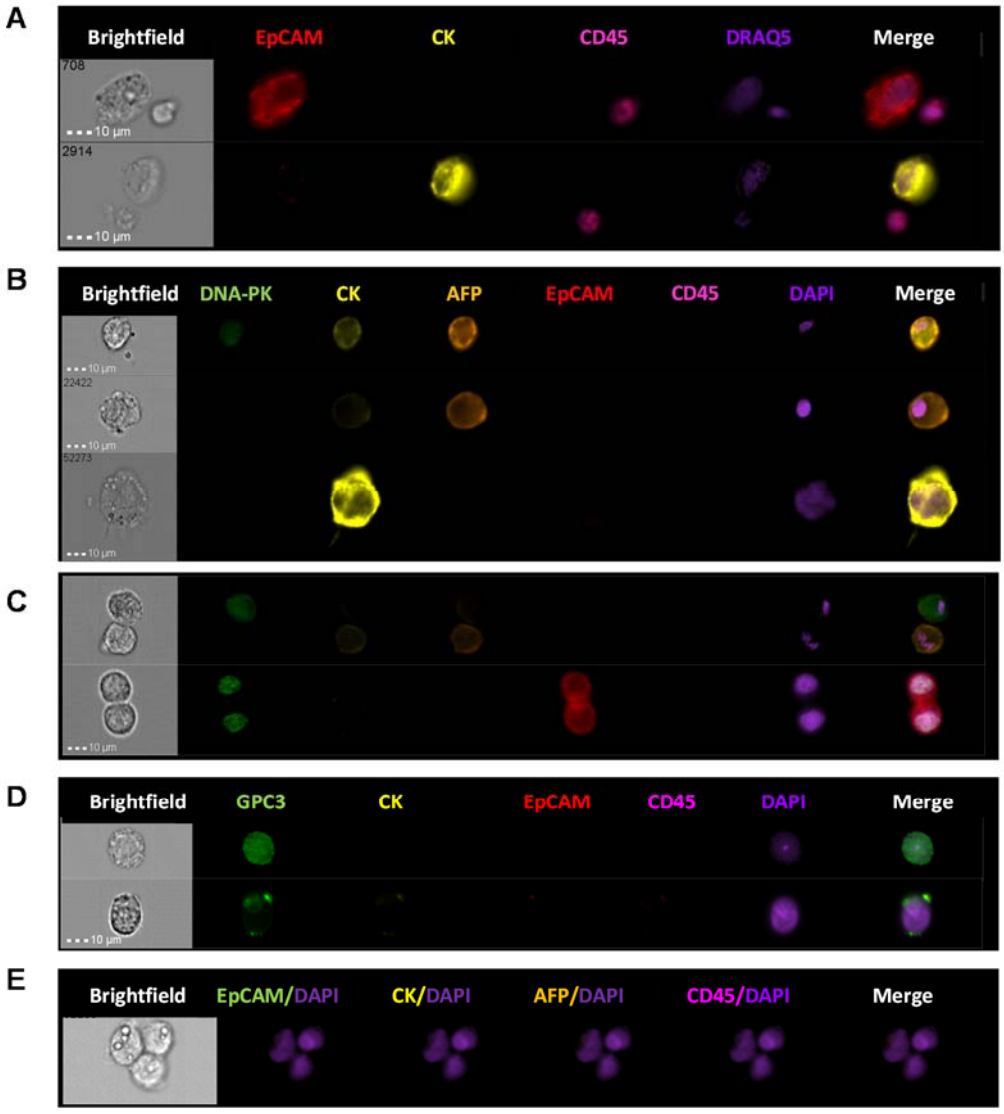
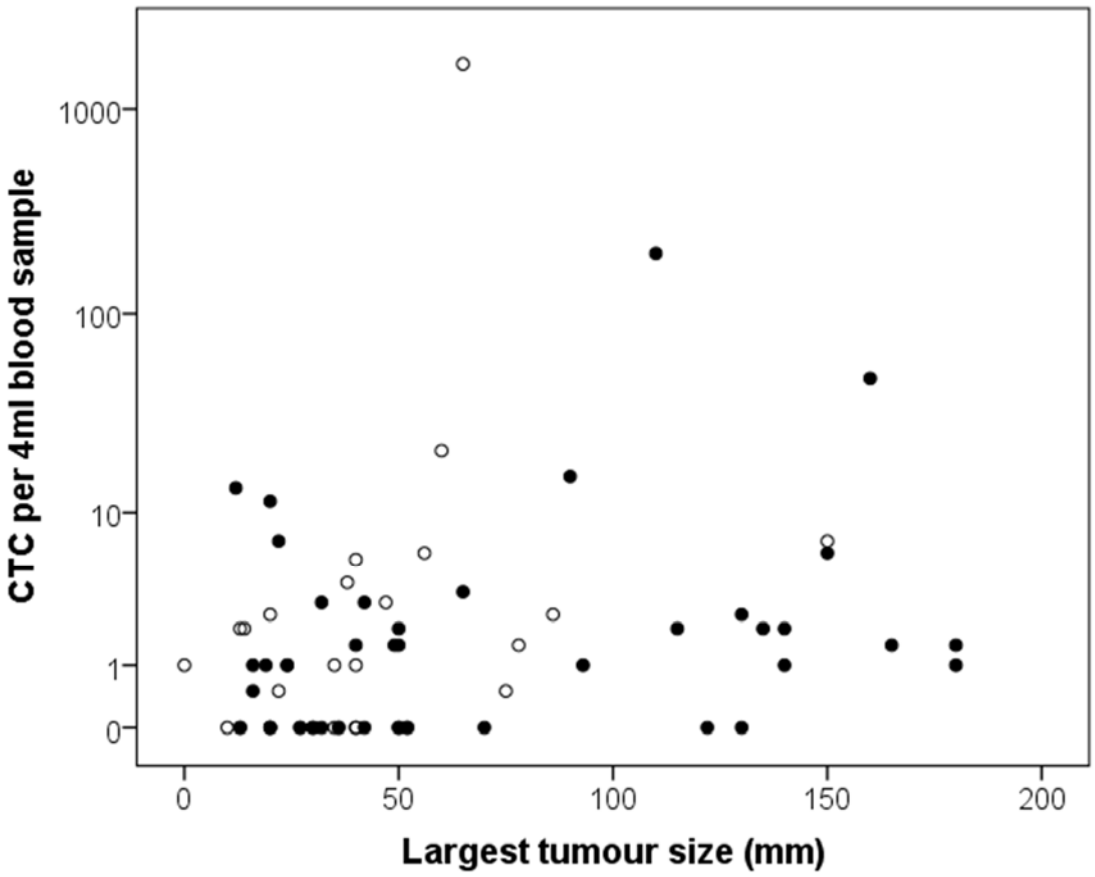
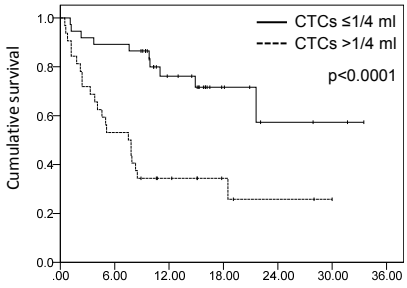


Figure 4

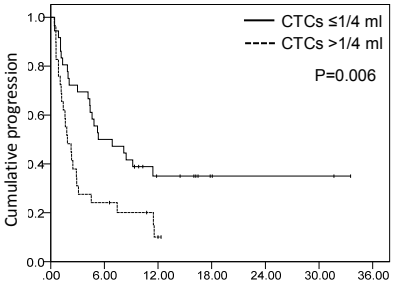

CTC per 4ml blood sample
1000
100
10
1
0
0
50
100
150
200
Largest tumour size (mm)

a)



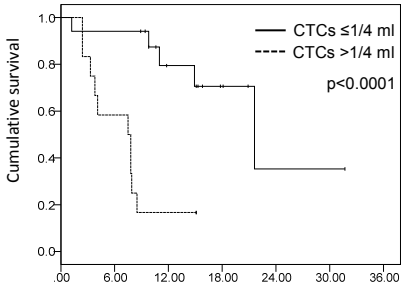
At-Risk Patients						
CTCs ≤1/4 ml	37	33	19	7	3	2
CTCs >1/4 ml	32	17	8	4	2	1

b)



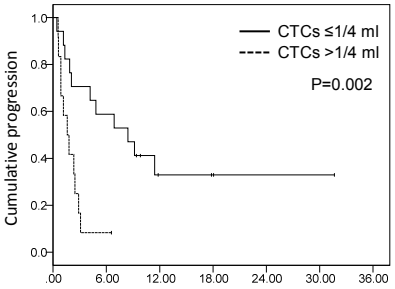
At-Risk Patients						
CTCs ≤1/4 ml	36	18	8	3	2	2
CTCs >1/4 ml	29	7	2	0	0	0

c)



At-Risk Patients						
CTCs ≤1/4 ml	17	16	9	4	4	1
CTCs >1/4 ml	12	7	2	0	0	0

d)



At-Risk Patients						
CTCs ≤1/4 ml	17	10	3	21	1	1
CTCs >1/4 ml	12	1	0	0	0	0

Figure 5. Additional CTC observations